



Vaccinia virus infection of mature dendritic cells results in activation of virus-specific naïve CD8⁺ T cells: A potential mechanism for direct presentation[☆]

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Abstract

Understanding how vaccinia virus (VV) generates immunity necessitates an appreciation for how this virus interacts with dendritic cells (DC), which are the most potent activators of naïve CD8⁺ T cells. In order to optimally activate naïve CD8⁺ T cells, DC must undergo maturation, during which costimulatory molecules are upregulated and cytokines are produced. In this report, we show that VV infection of immature murine bone marrow-derived DC (BMDC) failed to induce maturation. Similar results were obtained when CD8⁺ DC were analyzed, a subset shown previously to be important *in vivo* in the generation of a vaccinia-specific response. The finding that VV infection of DC resulted in APC that were incapable of initiating T-cell activation was surprising given the previously reported role for direct presentation in the generation of anti-VV CD8⁺ T-cell responses in mice. To address the potential mechanism responsible for direct presentation, we tested the hypothesis that previously matured DC were susceptible to vaccinia virus infection and could present newly synthesized VV-derived epitopes for CD8⁺ T-cell activation. Our results show, that during VV infection of mature DC, threshold levels of viral protein are produced that promote T-cell activation. These results suggest that, even though VV cannot mature DC, previously matured DC exposed to VV can generate a VV-specific CD8⁺ T-cell response providing a potential mechanism by which direct infection results in T-cell activation *in vivo*.

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Introduction

Although it has been almost 30 years since the eradication of smallpox (Henderson, 1976), there has been renewed interest in poxvirus pathogenesis because of the emerging threat of its use as an agent of bioterrorism (Henderson et al., 1999). Because of this threat, it is not only critical that we understand the pathogenesis of poxvirus infection, but also how immune responses are generated following infection. Vaccinia virus (VV) has been used extensively as a model for poxvirus infection (Kieny et al., 1984; Paoletti, 1996; Koszinowski and

Thomssen, 1975; Smith and Kotwal, 2002); nonetheless, a number of gaps remain in our understanding of the precise mechanism by which VV generates a protective cellular immune response. One key to understanding the generation of anti-poxvirus immunity is the elucidation of how poxviruses interact with dendritic cells (DC).

Dendritic cells play a key role in the initiation of a CD8⁺ T-cell response as they appear to be the antigen presenting cells that are capable of priming VV-specific CD8⁺ T cells *in vivo* (Banchereau and Steinman, 1998; Norbury et al., 2002). In order for DC to optimally prime naïve CD8⁺ T cells, DC must undergo a process termed maturation. Upon contact with an inflammatory stimulus such as TLR ligands, CD40 ligation, or cytokines (e.g. IL-1, type I IFN, or TNF- α), DC migrate from the site of antigen contact to a draining lymph node where they are poised to activate naïve T cells (Akira et al., 2000; Medzhitov, 2001; Caux et al., 1994; Wesa and Galy, 2001; Luft

Abbreviations: DC, dendritic cell; VV, vaccinia virus; BMDC, bone marrow-derived dendritic cell; UV-VV, UV-inactivated vaccinia virus.

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et al., 1998; Montoya et al., 2002; Sallusto and Lanzavecchia, 1994; Cumberbatch et al., 1991; Cumberbatch and Kimber, 1992). During this migration, DC upregulate the expression of costimulatory molecules CD80 and CD86 (also known as B7.1 and B7.2) which bind CD28 on naïve T cells in an interaction necessary for optimal T-cell activation (Harding et al., 1992; Harding and Allison, 1993; Freeman et al., 1993). Mature DC also secrete cytokines, such as IL-12 and type I IFN, that provide the additional signal necessary for the acquisition of CD8⁺ T-cell effector function (Curtsinger et al., 1999; Schmidt and Mescher, 1999; Schmidt and Mescher, 2002; Curtsinger et al., 2003; Curtsinger et al., 2005).

Previous studies of human DC have shown that VV fails to induce maturation and therefore costimulatory molecule expression (Engelmayer et al., 1999; Drillien et al., 2000). These studies also showed that VV replication in immature human DC is abortive. Importantly, inhibition of human DC maturation occurs well before the virus kills the cell, suggesting that, in addition to inducing death of infected DC, VV has mechanisms that can directly inhibit maturation. Moreover, infection of mature DC with VV only minimally altered costimulatory molecule expression and did not induce significant death (Engelmayer et al., 1999).

Vaccinia virus has a large arsenal of immune evasion proteins that counteract many of the anti-viral mechanisms of the innate and adaptive immune response. The VV proteins encoded by the K3L and E3L genes specifically affect the cell's response to dsRNA and therefore the expression and function of type I IFN, a potent cytokine for DC maturation (Beattie et al., 1991; Chang et al., 1992; Paez and Esteban, 1984; Xiang et al., 2002). Other proteins have been found to interfere with TLR signaling, an additional mechanism by which DC can be matured. The protein encoded by the A46R gene blocks TLR signaling by interacting with TLR adaptor proteins MyD88, Toll-IL-1 receptor domain-containing adaptor-inducing IFN- β (TRIF), and TRIF-related adapter molecule (TRAM) (Stack et al., 2005), while the A52R gene encodes a protein that has been implicated in blocking TLR signaling by blocking activation of NF- κ B, a key transcription factor involved in the induction of inflammatory gene transcription (Bowie et al., 2000; Harte et al., 2003; Sen and Baltimore, 1986b; Sen and Baltimore, 1986a; Baeuerle and Henkel, 1994). In addition, proteins encoded by the N1L and K1L genes are also able to inhibit activation of NF- κ B (DiPerna et al., 2004; Bartlett et al., 2002; Shisler and Jin, 2004). VV also produces receptor homologs for the cytokines type I IFN (encoded by the B18R gene) and IL-1 β (encoded by the B15R gene) (Symons et al., 1995; Colamonici et al., 1995; Smith and Chan, 1991). Each of these proteins can potentially prevent dendritic cell maturation and therefore have a profound effect on the generation of an adaptive immune response.

Because VV has so many ways of potentially inhibiting DC maturation, it may be difficult to imagine how DC infected with VV could directly present antigen to and activate naïve CD8⁺ T cells. However, direct presentation of viral antigen has been shown to play a significant role in the priming of an anti-VV CD8⁺ T-cell response in vivo (Basta et al., 2002; Shen et al.,

2002). Using a VV that contains genes encoding human cytomegalovirus proteins US2 or US11, both of which prevent direct presentation of endogenous VV antigens, Basta et al. showed that approximately 40% of the CD8⁺ T-cell response is due to direct presentation of VV antigens. Moreover, Shen et al. also used VV encoding US11 to show that anti-VV CD8⁺ T-cell responses generated with intraperitoneal and intravenous routes of infection were, for the most part, a result of direct presentation (Shen et al., 2002).

In these studies, we determined the effect of VV infection on mouse DC maturation. We found that VV did not induce mouse DC maturation as shown by the failure of bone marrow-derived DC (BMDC) or spleen-derived CD8⁺ DC to upregulate expression of the maturation markers. Interestingly, however, UV-inactivated VV (UV-VV) did induce upregulation of these costimulatory molecules as well as the secretion of inflammatory cytokines. As a result, UV-VV treated BMDC, but not VV-infected BMDC, were able to activate naïve CD8⁺ T cells in the presence of exogenous antigen. Infection of previously matured DC, while abortive, resulted in the presentation of adequate levels of newly synthesized viral protein for efficient T-cell activation. Thus, we propose that one mechanism by which direct presentation may occur in vivo is through virus infection of DC that were previously matured by a signal not associated with direct infection, e.g. cytokines or viral components released from neighboring infected cells.

Results

VV infection of immature mouse BMDC did not induce upregulation of costimulatory molecules

We first determined the effect of VV infection on the upregulation of costimulatory molecules CD40, CD80, and CD86 on murine DC. Mouse BMDC were infected with sucrose gradient purified VV-Ova at an MOI of 0.1, 1, or 10 for 24 h. We first looked at 24 h post-infection so that we could directly compare our results with those obtained by others in the human system. Expression of maturation markers on the infected cells was compared to DC treated with LPS or UV inactivated virus (UV-VV) for 24 h. As expected, treatment of mouse BMDC with LPS resulted in significant upregulation of CD40, CD80, and CD86 expression at 24 h (Fig. 1). However, infection of mouse BMDC with VV did not induce upregulation of any of these markers, regardless of MOI (Fig. 1). In contrast, treatment with UV-VV resulted in a dose-dependent increase CD40, CD80, and CD86 expression. CD40 was the least sensitive to upregulation by UV-VV, whereas CD86 expression was upregulated to a level that was greater than that observed with LPS. Of note, increased expression of these markers following treatment with UV-VV was absent at 8 h post-treatment but maximal at 18 h and thus was delayed compared to LPS-induced maturation (data not shown). It was possible that the kinetics with which VV-infection induced upregulation of these markers was delayed compared to LPS or alternatively that there was a transient upregulation of costimulatory markers early following infection that had returned to baseline by 24 h.

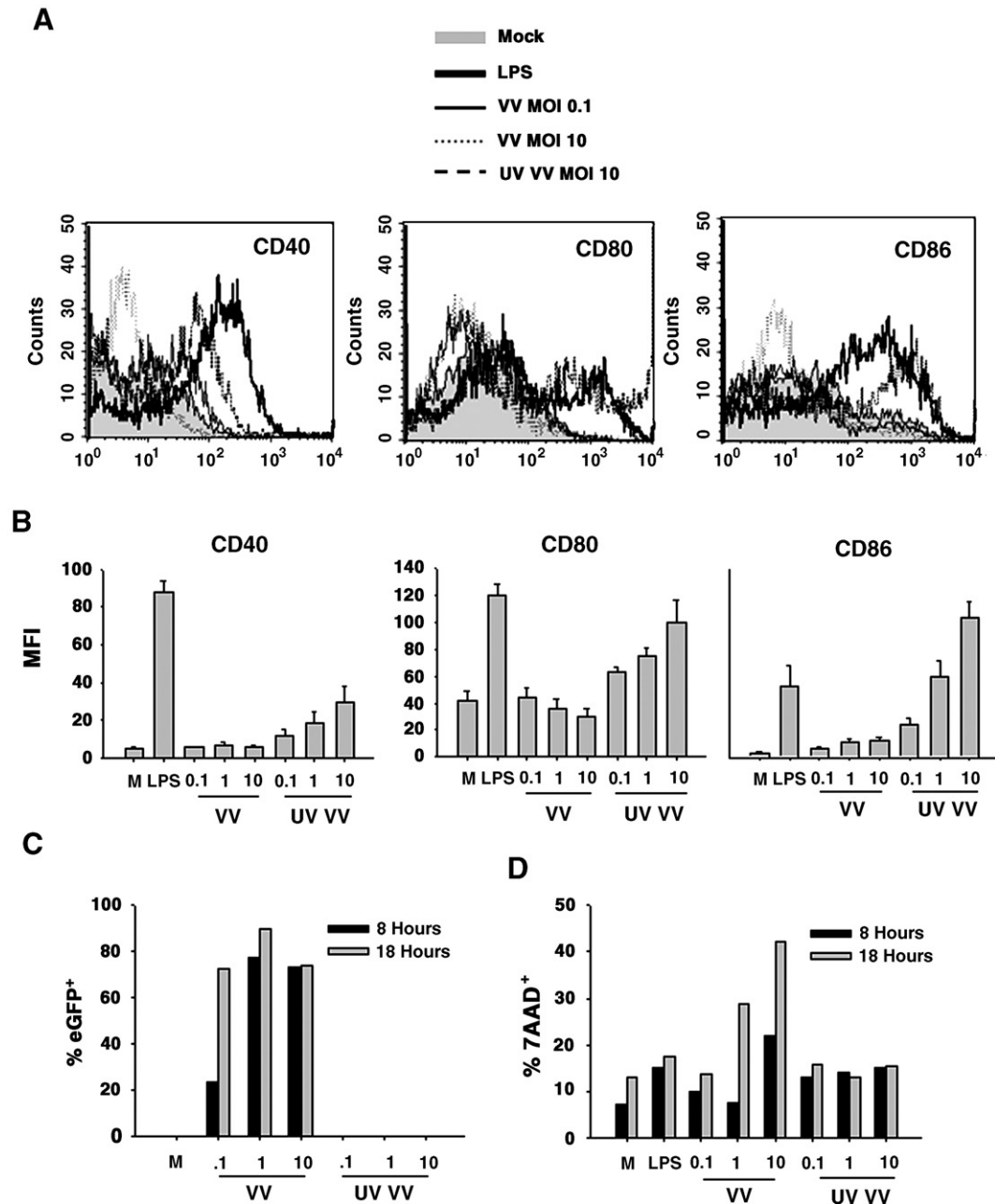


Fig. 1. VV infection fails to induce upregulation of maturation markers on murine BMDC. Mouse bone marrow cells were cultured in the presence of GM-CSF for 6 days and then treated with 300 ng/ml LPS, VV, or UV-VV for the indicated time or were mock-treated (M). The maturation phenotype of BMDC was determined by staining with anti-CD11c in combination with antibodies to the maturation markers CD40, CD80, and CD86. (A) Histograms showing CD40, CD80, and CD86 expression levels on CD11c⁺ BMDC that were mock-treated, LPS-treated, infected with VV (MOI 0.1 or 10), or treated with UV-VV (MOI 10) for 24 h. (B) Average geometric mean fluorescence intensities of CD40, CD80, and CD86 expression on BMDC treated for 24 h. (C) BMDC were infected with VV-eGFP and percent eGFP⁺ was measured at 8 or 18 h to determine infectivity. (D) BMDC were stained with 7AAD to assess death. Data are representative of at least 4 independent experiments. Cells in all analyses are following gating on the CD11c⁺ population.

However, this was not the case as analyses at 6 and 48 still showed a failure of VV to induce these molecules (data not shown). Together, these results show that UV-VV was a strong maturation stimulus, yet live virus was not capable of inducing maturation of mouse BMDC.

We next sought to determine the percentage of BMDC that became infected following exposure to VV. Experiments performed using a polyclonal anti-VV antibody following

infection with a vaccinia virus expressing eGFP (VV-EGF-POva) suggested that the antibody was not sensitive enough to detect the majority of infected cells. Thus, we performed these analyses with a VV expressing eGFP to quantitate infection. Importantly, VV-eGFP infection did not induce BMDC maturation, similar to the results seen with VV-Ova (data not shown). Using an MOI of 1 or 10, approximately 80% of BMDC were infected at 8 h as measured by eGFP expression

(Fig. 1C). At an MOI of 0.1, the number of infected cells at 8 h was less (20%) as would be expected. By 18 h post-infection with this low MOI, however, the percentage of infected cells increased to approximately 75%. No eGFP expression was detected in BMDC treated with UV-inactivated VV-eGFP. To determine the extent of cell death, we stained cells with 7AAD at 8 and 18 h post-infection. At 8 h pi, increased death was only seen after infection with the high MOI (Fig. 1D). By 18 h pi, there was a dose-dependent increase in the number of cells that were dying. As would be expected, there was no increase in death in cells that were treated with UV-inactivated virus.

VV infection of mouse BMDC fails to induce secretion of inflammatory cytokines

Another property associated with DC maturation is the ability to secrete pro-inflammatory cytokines. We used a cytometric bead array to determine the presence of IL-6, MCP-1, and TNF- α in the supernatants of mouse BMDC infected with VV or treated with LPS or UV-VV. In addition, we also determined the levels of IL-12p40 in BMDC supernatants by ELISA and IFN- α/β using a VSV-based bioassay. In these analyses, we found that infection with VV did not result in significant TNF- α , IL-12p40, or IFN- α/β secretion (Fig. 2). While BMDC infected with VV did secrete MCP-1, the level produced was much less than that secreted

by LPS-treated BMDC and was inversely proportional to MOI. A very low but detectable level of IL-6 was detected in BMDC infected with VV (MOI-10), but this value was minimal compared to that secreted by LPS-treated BMDC, and IL-6 secretion was not detected following infection at an MOI of 1 or 0.1.

In contrast, exposure of BMDC to UV-VV at an MOI of 10 induced detectable secretion of both TNF- α and IL-6, moderate production of MCP-1 and IL-12p40, and robust production of IFN- α/β . These results show that infection of BMDC with VV resulted in minimal cytokine production; however, treatment with UV-VV resulted in production of a similar profile of cytokines as compared to LPS-treated cells, albeit at lower levels. The exception to this was IFN- α/β , where the level exceeded that of LPS-treated cells.

Vaccinia infection of CD8⁺ DC results to a failure to mature

A recent study has shown that CD8⁺ DC are the predominant DC that present VV antigen in vivo following infection by the subcutaneous or intravenous routes (Belz et al., 2004). Given this, we investigated the effect of vaccinia infection on CD8⁺ DC as it was possible that the result of VV infection of this DC subset was distinct from that observed in myeloid DC. As a method for the in vitro differentiation of bona fide CD8⁺ DC is lacking, we isolated splenic CD8⁺ DC for our studies. Isolated

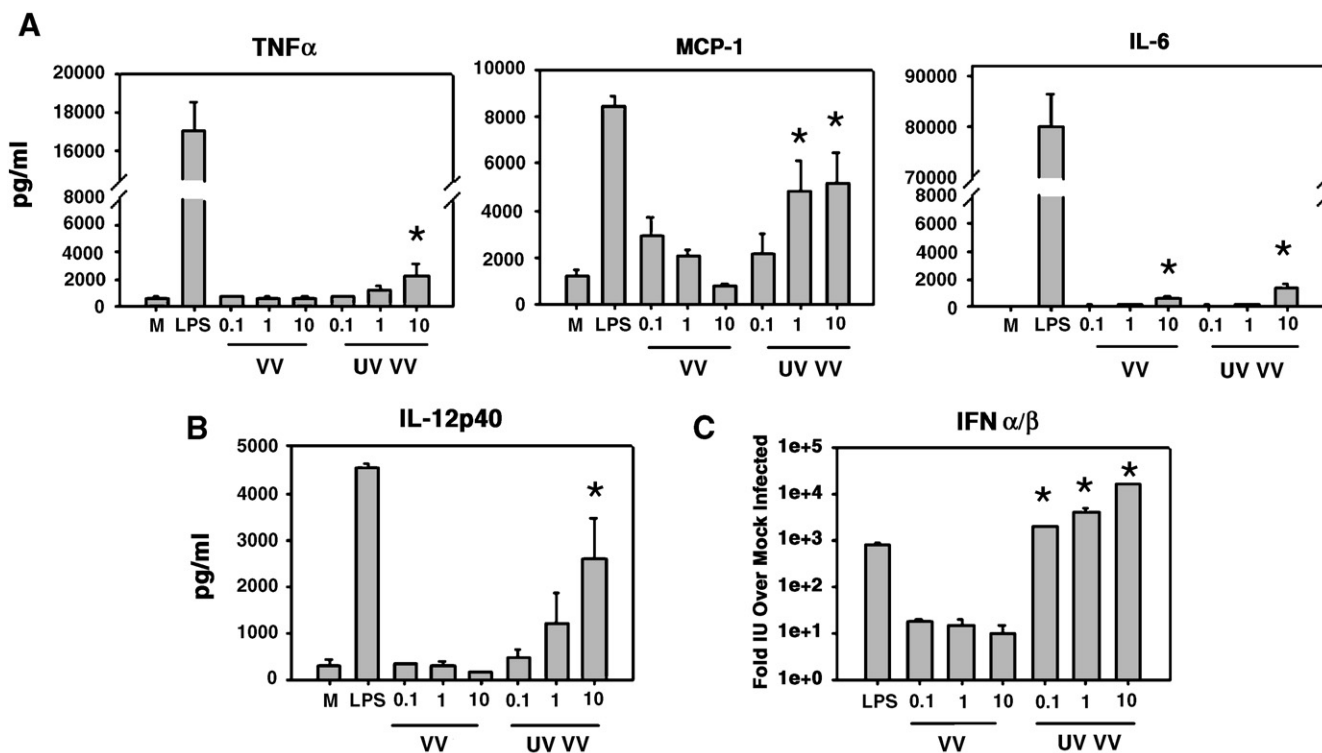


Fig. 2. VV infection fails to induce inflammatory cytokine secretion in murine BMDC. Supernatants from treated BMDC were harvested at 24 h and analyzed for the presence of inflammatory cytokines. (A) Levels of TNF- α , MCP-1, and IL-6 in BMDC supernatants as measured by CBA (see Materials and methods). (B) IL-12p40 levels in BMDC supernatants were measured by ELISA. (C) Type I IFN levels in BMDC supernatants relative to mock-treated BMDC as measured by the VSV-based IFN- α/β bioassay. Levels of IFN- α/β in polyI:C treated BMDC supernatants were similar to LPS. * $P < 0.05$ compared with mock-infected BMDC (A and B) or compared with VV-infected BMDC (C). Values are the average of 3 experiments.

DC were infected with recombinant vaccinia virus expressing eGFP (VV-EGFP^{Ova}) at increasing MOI, and viability and maturation were assessed at 12 h. This virus also allowed direct determination of infected cells. As expected, there was a dose-dependent increase in the percent of eGFP⁺ CD8 α ⁺ DC 12 h pi (Fig. 3A). VV infection did not result in death at levels significant above mock infection CD8 α ⁺ DC. However, others have noted high levels of nonspecific death in uninfected splenic CD8 α ⁺ DC after overnight culture (Rizzitelli et al., 2005) and thus the death of infected cells is hard to assess above this background (Fig. 3B). As has been shown previously (Rizzitelli et al., 2005), isolated CD8⁺ DC cultured overnight upregulated CD86 compared to cells tested directly ex vivo (data not shown). Addition of LPS resulted in further upregulation of this maturation marker (Fig. 3). When CD86 expression was analyzed on the infected cells, a significantly reduced maturation was observed, consistent not only with a failure of vaccinia virus infection to induce maturation, but also a block in the spontaneous maturation that results from overnight culture. At 18 h pi, CD8 α ⁺ DC treated with LPS

have even further upregulated CD86 expression, while VV-EGFP^{Ova}-infected cells still exhibit an immature phenotype (data not shown). Of note, a small fraction of cells do undergo limited maturation. These cells, however, are a very small percentage of the starting DC population. Together, these data suggest that, similar to the in vitro generated myeloid DC, VV fails to mature the vast majority of CD8⁺ DC.

VV-infected BMDC fail to activate naïve CD8⁺ T-cell proliferation and function

As it is well established that the increased expression of costimulatory molecules and the secretion of inflammatory cytokines are essential for DC to effectively prime a CD8⁺ T-cell response (Harding et al., 1992; Harding and Allison, 1993; Freeman et al., 1993; Curtsinger et al., 1999; Curtsinger et al., 2003; Schmidt and Mescher, 1999; Schmidt and Mescher, 2002; Curtsinger et al., 2005), we tested the ability of VV-infected DC to activate naïve CD8⁺ T cells. Given the similarity in the maturation of CD8⁺ DC and the BMDC following infection with VV, we used BMDC for these assays because of the difficulty in obtaining adequate numbers of CD8⁺ DC for our studies. Treated BMDC were co-cultured with LCMV gp_{33–41} specific CD8⁺ T cells from P14 TCR transgenic mice. Use of this system allowed us to determine the functional significance of changes in the maturation state of the DC that resulted from VV exposure while keeping constant the amount of exogenous peptide (LCMV gp_{33–41}). Fig. 4A shows a representative experiment from our analyses of CFSE-labeled LCMV gp_{33–41}-specific CD8⁺ T cells that were cultured for 3 days with BMDC previously treated with LPS, VV, or UV-VV. Fig. 4B and C shows the averages from 3 independent experiments. At limiting concentrations of gp_{33–41} peptide (10^{–10}M), LPS-treated BMDC induced significant proliferation and function of naïve gp_{33–41}-specific CD8⁺ T cells. On average, approximately 70% of cells entered division following stimulation with BMDC matured by exposure to LPS, and of the dividing cells at least 65% produced IFN- γ .

In contrast, BMDC infected with VV induced very minimal proliferation of naïve gp_{33–41}-specific CD8⁺ T cells; the percentage of cells that had divided following co-culture with BMDC infected with an MOI of 0.1 or 1 was similar to that observed following culture with mock-infected cells. For cells infected with the highest MOI (10), the percent divided was below that of mock-infected BMDC when the 10^{–9} M peptide concentration was used. Furthermore, CD8⁺ T cells cultured with VV-infected BMDC pulsed with limiting peptide concentrations were not functional as shown by their failure to produce IFN- γ following peptide stimulation. Mock-treated BMDC or those infected with VV were all similar in their ability to promote the acquisition of effector function in naïve T cells. Of note, at high concentrations of peptide (10^{–8} M), T cells co-cultured with VV-infected BMDC underwent proliferation and gained function to a level similar to that observed following culture with LPS-treated BMDC, demonstrating that the failure of T cells to proliferate when co-cultured with VV-infected BMDC was not the result of the presence of virus that infected

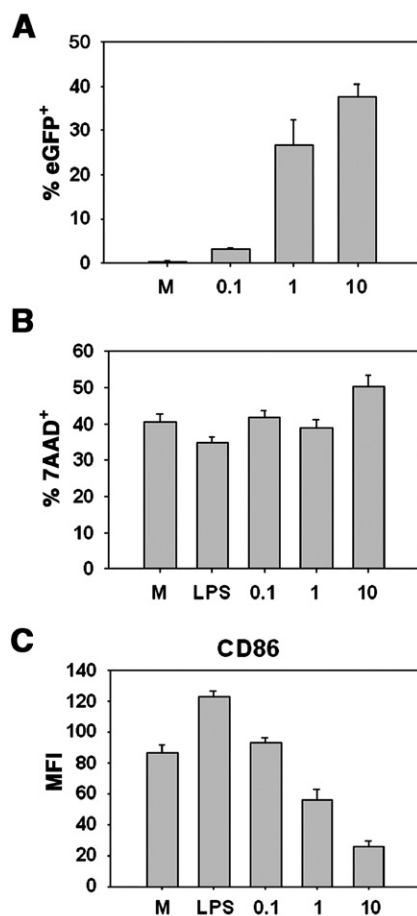


Fig. 3. Infection of CD8⁺ DC with vaccinia virus fails to induce maturation. CD8⁺ DC enriched from the spleen of C57BL/6 mice were infected with VV-EGFP^{Ova} (MOI=0.1, 1, or 10) for 12 h. Cells were then stained with antibodies to CD11c, CD8, and CD86 or with 7AAD. Data are obtained following gating on the CD11c⁺ CD8⁺ cells in the live cell gate at 12 h post-infection. (A) Percent eGFP⁺, (B) percent 7AAD⁺, and (C) MFI of CD86 expression are shown. Data are the average of 3 experiments.

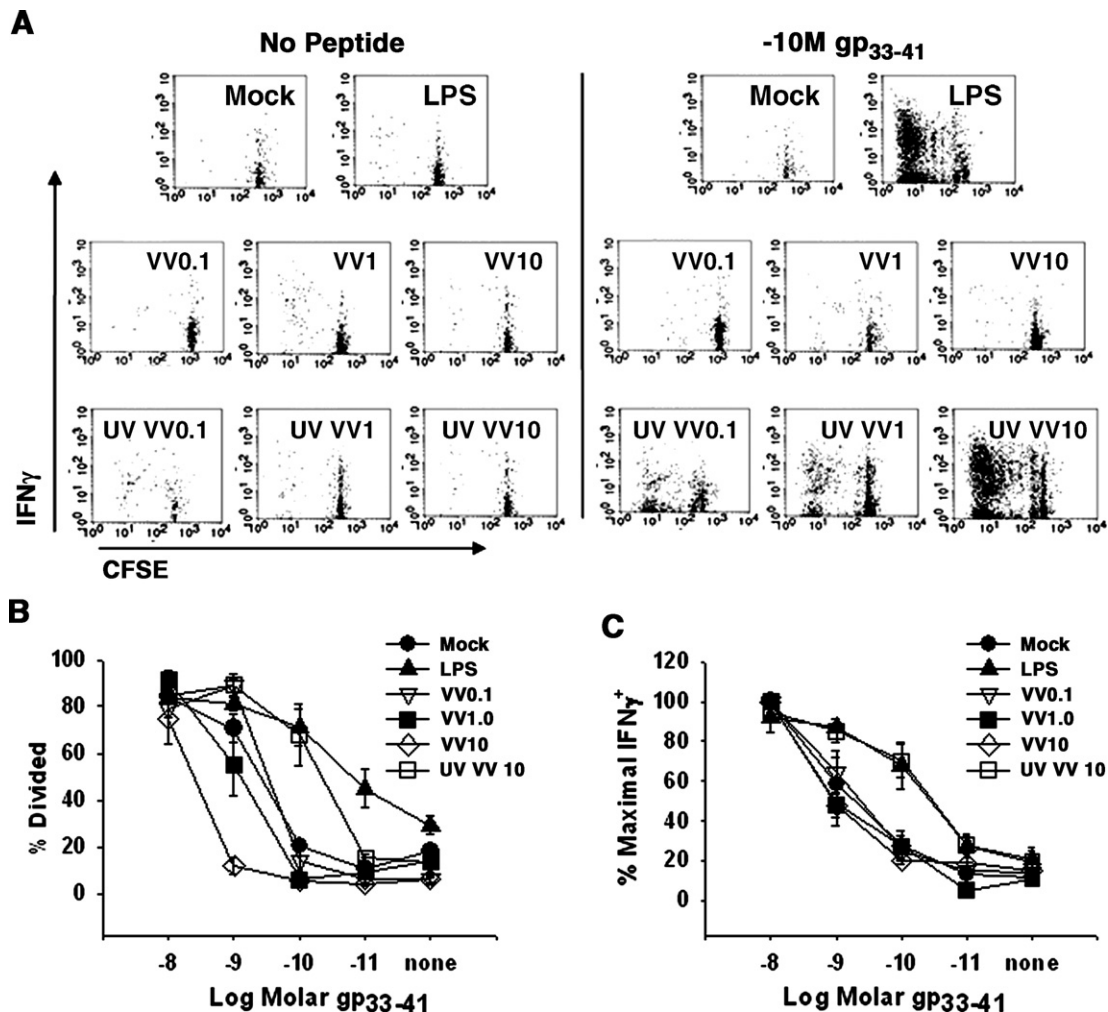


Fig. 4. VV-infected murine BMDC do not activate naïve CD8⁺ T cells in the presence of limiting concentrations of exogenous peptide antigen. Mouse BMDC were treated with LPS or UV VV or infected with VV for 24 h. DC were then pulsed with titrated concentrations of LCMV gp₃₃₋₄₁ peptide. Pulsed BMDC were washed and co-cultured with CFSE-labeled splenocytes from naïve Rag^{-/-} gp₃₃₋₄₁-specific P14 TCR transgenic mice for 3 days. (A) Proliferation and function of P14 CD8⁺ T cells after co-culture with unpulsed or 10⁻⁸ M pulsed BMDC treated under the designated conditions. (B) Percent of P14 CD8⁺ T cells that divided after co-culture with BMDC pulsed with varying concentrations of gp₃₃₋₄₁ peptide. (C) Percent maximal IFN- γ production by divided P14 CD8⁺ T cells after co-culture with BMDC pulsed with titrated concentrations of gp₃₃₋₄₁ peptide. BMDC infected with VV (MOI=0.1, 1, or 10) or UV VV (MOI=10) are shown. Values in B and C are the average of 3 experiments.

the T cells or another negative signal that prevented T-cell activation.

Similar to LPS-treated cells, BMDC exposed to UV-VV induced T-cell proliferation and function. Proliferation was similar in both the percentage of cells that entered division and the proliferation index, which is a measure of the average number of divisions undergone by the T cells that entered division ($2.6 \pm \text{SE } 0.46$ for LPS and $2.7 \pm \text{SE } 0.27$ for UV-VV). Additionally, a similar percentage of CD8⁺ T cells cultured with BMDC treated with UV-VV produced IFN- γ compared with cultures stimulated with LPS-matured BMDC (70% vs. 68%) (Fig. 4C). This is consistent with our results from Figs. 1 and 2 showing that BMDC treated with UV-VV at an MOI of 10 exhibited high expression of the costimulatory molecules CD80 and CD86 and secreted inflammatory cytokines. Together, these data show that VV infection rendered BMDC incapable of stimulating naïve CD8⁺ T-cell proliferation and function at limiting concentrations of peptide antigen that were capable of

promoting high levels of proliferation and function when LPS-matured or UV-VV-matured DC were used as APC.

VV-infected BMDC exhibit decreased antigen presentation

While our data from Figs. 1 and 2 would suggest that the failure of VV-infected DC to activate naïve T cells was because of the lack of costimulatory molecule expression, it was possible that VV infection resulted in decreased antigen presentation following peptide loading that also contributed to this effect. To address this issue, BMDC infected with VV or matured with LPS were pulsed with varying concentrations of LCMV gp₃₃₋₄₁ peptide. BMDC were then co-cultured with an established gp₃₃₋₄₁-specific CTL line and IFN- γ production assessed as a readout for peptide presentation. LPS-treated BMDC induced half-maximal IFN- γ production at approximately a half-log lower concentration of antigen compared to mock-infected BMDC. This is consistent with previous studies

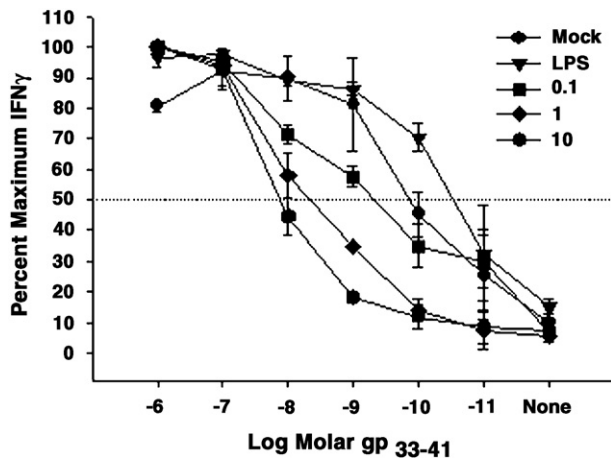


Fig. 5. Decreased antigen presentation by VV-infected BMDC. Mouse BMDC were mock-treated, treated with LPS, or infected with VV (MOI=0.1, 1, or 10) for 18 h and then pulsed with varying concentrations of gp₃₃₋₄₁. Following washing to remove unbound peptide, BMDC were co-cultured with a gp₃₃₋₄₁-specific CTL line for 5 h in the presence of GolgiPlug. IFN- γ production was determined by FACS analysis and expressed as percent maximal IFN- γ production. Data are the average of 3 independent experiments.

showing that MHC class I expression is increased on mature DC (Kukutsch et al., 2000; Li et al., 2001; MacAry et al., 2001). Infection with VV resulted in a dose-dependent increase in the amount of peptide required to induce half-maximal IFN- γ production in the CTL lines, in that the higher the MOI, the greater the amount of peptide required (Fig. 5). This suggested that VV infection resulted in a partial block in antigen presentation in the context of MHC class I on the surface of BMDC.

While VV infection resulted in a decrease in antigen presentation in BMDC, this does not fully explain the inability of VV-infected BMDC to activate naïve CD8⁺ T cells in the presence of limiting concentrations of exogenous antigen. The pattern of induction of proliferation in naïve gp₃₃₋₄₁-specific CD8⁺ T cells followed the pattern of antigen presentation as readout by the CTL line, suggesting that the level of presented peptide is playing a large role in controlling the entry of cells into division. However, this is not the case with elicitation of effector function since mock- and VV-infected BMDC were equally incapable of eliciting IFN- γ production from naïve CD8⁺ T cells, despite the fact that mock-infected BMDC were technically presenting more peptide (Fig. 4C). This result suggested that the lack of costimulatory molecule expression was playing a more dominant role in the acquisition of effector function in this assay, although peptide levels may still be contributing to the effect. These data make the important point that vaccinia virus utilizes a number of mechanisms to undermine generation of the immune response.

Mature BMDC infected with VV are capable of activating naïve CD8⁺ T cells

It has been shown that a significant amount (~40%) of the CD8⁺ T-cell response to VV in vivo is due to direct priming of

naïve CD8⁺ T cells by infected DC (Basta et al., 2002). Our findings raised the question of how this could occur if infected DC undergo maturation as has been shown to be necessary for efficient naïve CD8⁺ T-cell activation. We hypothesized that DC matured via an alternative signal could become infected with VV leading to presentation of viral antigen. To test this possibility, BMDC were treated with LPS for 20 h, a period of time which should allow for maximal maturation. Matured DC were then infected with VV for 12 h, and the expression of CD40, CD80, and CD86 was determined. We found that BMDC pre-treated with LPS retained high expression of these markers following infection with VV at an MOI of 0.1, 1, and 10 (data not shown). We also found that VV did not induce significant death (as shown by staining for active caspase 3) of BMDC pre-treated with LPS (data not shown). These results showed that VV did not exhibit cytopathic effects and had minimal impact on maturation marker expression on murine DC that were mature at the time of infection. When mature BMDC were infected with VV-eGFP at MOIs of 10, 1, or 0.1–10%, 12% and 5% of cells were eGFP⁺ at 20 h pi, in stark

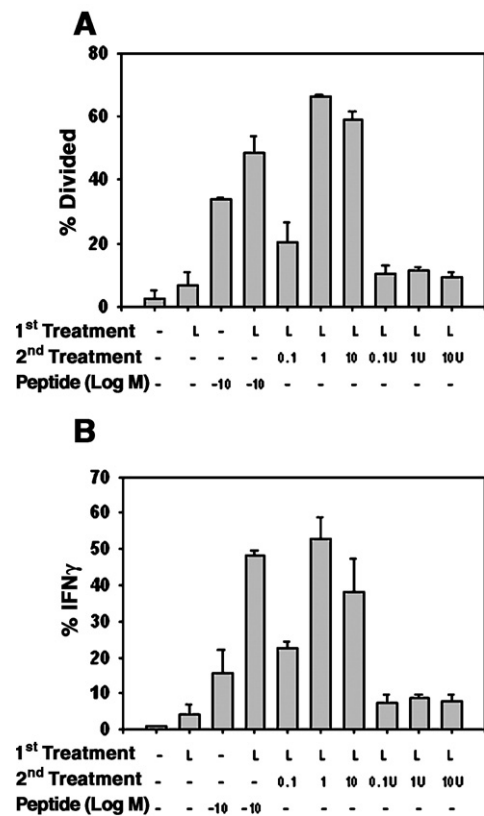


Fig. 6. dMurine BMDC previously matured with LPS and infected with VV-gp₃₃₋₄₁ are capable of activating naïve P14 gp₃₃₋₄₁-specific CD8⁺ T cells. Mouse BMDC were treated with LPS (L) for 18 h followed by pulsing with 10⁻¹⁰ M gp₃₃₋₄₁, infection with VV-gp₃₃₋₄₁, or exposure to UV VV-gp₃₃₋₄₁ for 12 h. Virus treatments were performed with MOIs of 0.1, 1, and 10. Treated BMDC were co-cultured for 3 days with CFSE-labeled splenocytes from naïve gp₃₃₋₄₁-specific P14 TCR transgenic mice. (A) Percent of gp₃₃₋₄₁-specific CD8⁺ T cells that entered division. (B) Percent of P14 T cells that produced IFN- γ following restimulation at the end of the three-day co-culture period. Values are the average of 3 experiments.

contrast to immature BMDC (MOI=10), where approximately 80% were eGFP⁺ at these time points (data not shown). These data suggest that mature BMDC are relatively non-permissive to infection.

We next determined whether the previously matured mouse BMDC infected with VV were capable of activating naïve CD8⁺ T cells. To test this possibility, BMDC previously matured by LPS treatment were infected with VV-gp_{33–41} and cultured with naïve P14 gp_{33–41}-specific CD8⁺ T cells for 3 days (similar to what was done in Fig. 4) (Fig. 6). Proliferation and function of these CD8⁺ T-cell populations were compared to naïve P14 T cells cultured with mock or LPS-treated BMDC pulsed with 10^{−10} M gp_{33–41}. BMDC matured with LPS and subsequently infected with VV-gp_{33–41} (MOI=1 or 10) were capable of inducing proliferation and function of P14 T cells similar to LPS-matured BMDC pulsed with 10^{−10} M gp_{33–41} (Fig. 6). Similar percentages of cells entered division and the average number of divisions the T-cell population underwent (division index) were similar (1.3±0.1 (MOI=1), 1.2±0.1 (MOI=10), and 1.7±0.1 (LPS)). As expected, LPS-matured BMDC treated with UV-VV did not induce proliferation or function of P14 CD8⁺ T cells, demonstrating the requirement for VV protein expression following infection in order for presentation of gp_{33–41} to occur. Furthermore, no activation was apparent when LPS-matured BMDC infected with VV-VPE16, a recombinant VV expressing the HIV gp160 protein was used (data not shown). Together, these data suggest that VV protein, albeit at low levels, is being produced in previously matured BMDC exposed to VV. Moreover, mature DC exposed to VV are capable of activating naïve CD8⁺ T cells to proliferate and acquire effector function.

Discussion

Previous studies in mice suggested that DC are the relevant APC for priming a VV-specific CD8⁺ T-cell response *in vivo* (Banchereau and Steinman, 1998; Norbury et al., 2002). In this report, we investigated the capacity of VV-infected murine DC to undergo maturation as a result of infection and subsequently to activate naïve CD8⁺ T cells. We demonstrated that VV-infected mouse BMDC did not undergo maturation as measured by the upregulation of costimulatory markers and the production of cytokines and were thus not capable of activating naïve CD8⁺ T cells in the presence of limiting concentrations of exogenous antigen. The majority of our analyses were performed using immature BMDC, which are predominantly of myeloid phenotype, as large numbers of these cells can be readily generated. However, as a recent study suggested that CD8 α ⁺ DC are the predominant activators of vaccinia-specific CD8⁺ T cells *in vivo* following infection by the intravenous or subcutaneous routes (Belz et al., 2004), we also tested the ability of vaccinia infection to induce maturation of CD8 α ⁺ DC isolated from the spleen. As with the *in vitro* generated myeloid DC, CD8 α ⁺ DC infected with vaccinia virus failed to undergo maturation. In our analysis of the infected cells that survived after overnight culture, we did observe a small percentage of cells that underwent limited maturation. Given the small

percentage of the initial population that these would have constituted, one possibility is that these cells were farther along in their maturation at the time of infection, thereby limiting the cytopathic effect of the virus as was observed for myeloid DC. However, determining whether this is the case will require further investigation. Nonetheless, in general, the effect of VV infection on the maturation of myeloid and CD8⁺ DC appeared similar.

Previous studies in mice have shown that direct presentation by infected APC contributes significantly to the generation of the VV-specific CD8⁺ T-cell response (Basta et al., 2002; Shen et al., 2002). This finding was seemingly contradictory to our studies showing that infection with VV failed to trigger DC maturation to become proficient activators of naïve T cells. Of note, the failure to undergo maturation was observed at times post-infection between 6 and 48 h (data not shown and Fig. 1), encompassing the timeframe during which Norbury et al. reported presentation of vaccinia virus proteins in the draining lymph node (Norbury et al., 2002).

Given the failure of VV-infected immature DC to activate CD8⁺ T cells, we tested the possibility that previously matured DC were susceptible to infection with VV and could thereby act directly as APC capable of activating naïve CD8⁺ T cells. In support of this hypothesis, we found that BMDC matured by LPS and subsequently infected with VV-gp_{33–41} retained expression of maturation markers and were able to activate naïve gp_{33–41}-specific CD8⁺ T cells. This ability of previously matured DC to present vaccinia peptides is in agreement with a recent report from Wilson et al., which showed that previously matured dendritic cells could present viral antigen following HSV infection (Wilson et al., 2006). Hence, the ability of infected, mature DC to directly present viral antigen at a level adequate for naïve T-cell activation may provide a potential mechanism to reconcile the seemingly contradictory findings that direct presentation can occur *in vivo* and that infection of immature DC is cytopathic and fails to induce maturation. While it remains a formal possibility that cross-presentation could contribute to the T-cell activation observed in our infected mature BMDC cultures, we believe that this is highly unlikely given the following: (1) previously matured BMDC make very little viral protein, limiting the amount of antigen available for cross-presentation, (2) the infection is not cytopathic also limiting the antigen source and (3) mature BMDC are not efficient at cross-presentation (Hotta et al., 2006).

While the possibility of previously matured DC becoming infected with VV and directly priming naïve CD8⁺ T cells is attractive, we realize that additional scenarios are possible following infection with VV *in vivo* that could also contribute to direct priming of CD8⁺ T cells. One possibility is that VV can mature DC of a different type, such as plasmacytoid DC. However, although these DC have been shown to produce copious amounts of IFN- α / β that may promote maturation of neighboring DC, their ability to prime naïve T cells *in vivo* remains controversial (Facchetti et al., 1988; Siegal et al., 1999; Nakano et al., 2001; Asselin-Paturel et al., 2001; Krug et al., 2003; Schlecht et al., 2004).

While infection with VV did not induce BMDC maturation, treatment of BMDC with UV-inactivated virus resulted in upregulation of CD40, CD80, and CD86 as well as the production of a number of cytokines. Upregulation of CD86 by UV-inactivated virus is in agreement with results observed for human *in vitro* generated DC (Drillien et al., 2004). This result opens the door to the possibility that vaccinia virus contains a TLR agonist, as has been reported for a number of other viruses such as measles virus (TLR2), respiratory syncytial virus, and mouse mammary tumor virus (both TLR4) (Bieback et al., 2002; Kurt-Jones et al., 2000; Rassa et al., 2002). Alternatively, a non-TLR mediated event may be responsible for the maturation signal. Further studies are required to discriminate among these possibilities.

As previously described, VV encodes for a number of immune evasion proteins, namely cytokine receptor homologues and inhibitors of TLR signaling (Beattie et al., 1991; Chang et al., 1992; Paez and Esteban, 1984; Xiang et al., 2002) (Stack et al., 2005) (Bowie et al., 2000; Harte et al., 2003; Sen and Baltimore, 1986b; Sen and Baltimore, 1986a; Baeuerle and Henkel, 1994; DiPerna et al., 2004; Bartlett et al., 2002; Shisler and Jin, 2004; Symons et al., 1995; Colamonici et al., 1995; Smith and Chan, 1991). In VV-infected BMDC supernatants, we could not detect significant levels of TNF- α , IFN- γ , and functional IFN- α/β . Certain strains of VV do produce a cytokine receptor homologue for TNF- α ; however, the WR strain used in our studies is not among them (Upton et al., 1991; Howard et al., 1991). Furthermore, while the WR strain does produce an IFN- γ cytokine receptor homologue, studies have shown that it has a very low binding affinity for mouse IFN- γ (Alcami and Smith, 1995; Mossman et al., 1995). Hence, we do not believe that the inability to detect these two cytokines is due to a masking effect of any VV-derived cytokine binding protein. The WR strain of VV does produce a cytokine receptor homologue for type I IFN, which may contribute to the inability of VV to induce DC maturation and the inability of supernatants from infected BMDC to protect from VSV-mediated cytolysis in our bioassay (Symons et al., 1995; Colamonici et al., 1995).

Previous studies with the human system have shown that cytopathic effects in VV-infected DC are delayed out to 48 h post-infection. It was then reasoned that the inability of VV to mature human DC was due to immune evasion proteins. In our studies with VV-eGFP, we observed that, by 18 h post-infection with an MOI of 10, most BMDC were infected and undergoing apoptosis as shown by caspase 3 activation and 7AAD staining (Fig. 1 and data not shown). However, at 8 h post-infection, before the observation of widespread cytopathic effects, we see no increase in the expression of maturation markers in infected or uninfected cells (data not shown). These results leave open the possibility that VV immune evasion proteins can play a role in the failure to induce DC maturation at early times before the initiation of cell death. Alternatively, the ability of vaccinia virus infection to induce maturation may simply be kinetically delayed compared to maturation signals like LPS (as would be suggested by the increase in maturation markers seen at 8 h

post-treatment of BMDC with LPS, but not UV-VV). In support of this, infection of BMDC followed by LPS treatment 8 h later appeared to “rescue” the BMDC, resulting in maturation marker expression similar to in pattern, but lower in magnitude compared to LPS alone. Not surprisingly, LPS treatment following 24-hour infection did not induce any maturation (data not shown).

Because the mouse is used as a model for the human anti-VV immune response, it is important that its use be validated to the greatest extent possible. One area of importance is the regulation of DC maturation following infection. In our studies, we found many parallels in the results of VV infection of murine and human DC. Furthermore, these studies provide novel insights into the outcome of infection of CD8⁺ DC, a subset not previously analyzed. Infection of both human and murine DC results in cytopathic effects. Also in both cases there is a failure to induce upregulation of costimulatory molecules. Together, these findings provide support for the use of this model in understanding the generation of protective responses following infection with vaccinia virus.

In summary, our data show that vaccinia virus is incapable of triggering maturation of *in vitro* differentiated murine BMDC or splenic CD8⁺ DC following infection and as a result infected DC are inefficient activators of naïve CD8⁺ T cells. However, VV can initiate infection of mature DC that, even though abortive, allows for the production of a low level of viral protein that is adequate for the activation of naïve CD8⁺ T cells. In contrast to infectious virus, exposure to UV inactivated virus induced robust DC maturation suggesting that viral components contain a DC maturation signal. Thus, one possibility is that viral products released from infected cells or other inflammatory signals produced during vaccinia infection promote DC maturation. Matured DC subsequently abortively infected with VV could then potentially activate virus-specific CD8⁺ T cells *in vivo*. These results provide new insights into the regulation of both DC maturation and CD8⁺ T-cell activation as a result of poxvirus infection.

Materials and methods

Mice and cell lines

C57BL/6 mice were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Rag^{-/-} P-14 lymphocytic choriomeningitis virus (LCMV) gp33–41-specific T-cell receptor (TCR) transgenic mice on a C57BL/6 background were purchased from Taconic. NTCC L929 is a fibroblast cell line derived from C3H/An (*H-2^k*) mice grown in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone, Logan, UT), L-glutamine, sodium pyruvate, nonessential amino acids, HEPES, penicillin, streptomycin (BioWhittaker, Walkersville, MD), and 5×10^{-5} M 2-mercaptoethanol. All research performed on mice in this study complied with federal and institutional guidelines set forth by the Wake Forest University Animal Care and Use Committee.

Generation of bone marrow-derived dendritic cells

The protocol used to generate BMDC was similar to that used by Inaba et al. (1992). Bone marrow was harvested from the femurs and tibias of mice, and RBC were lysed with ammonium chloride buffer (BioWhittaker, Walkersville, MD). Bone marrow cells were then plated at 5×10^5 cells/well in a 24-well plate in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone, Logan, UT), HEPES, gentamicin sulfate (BioWhittaker, Walkersville, MD), 5×10^{-5} M 2-mercaptoethanol, 50 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (Biosource, Camarillo, CA). Every 2 days, the cell culture medium of the BMDC cultures was removed followed by the addition of fresh medium containing 50 ng/ml recombinant GM-CSF.

Isolation of CD8⁺ DC from the spleen

Minced spleens from C57BL/6 mice were incubated in the presence 100 μ g/ml Collagenase D (Roche, Indianapolis, IN) for 30 min at 37 °C. The tissue was then passed through a 70 μ m filter. CD11c⁺ cells were enriched by negative selection using the using Miltenyi microbeads as per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). In some cases, CD8⁺ DC were subsequently isolated by positive selection.

Viruses

The recombinant vaccinia viruses expressing ovalbumin, VV-Ova, and VV-EGFP-Ova were a kind gift from J. Bennink (NIH). VV-vPE-16 used as a control virus is a recombinant vaccinia virus construct that expresses the gp160 protein from the IIIB strain of human immunodeficiency virus and was a kind gift of Patricia Earl and Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD) (Earl et al., 1991). VV-gp_{33–41}, a recombinant VV expressing the lymphocytic choriomeningitis virus gp_{33–41} epitope, was a kind gift from J.M. Grayson and was prepared as described previously (Whitton et al., 1993). VV were sucrose gradient purified prior to infection of dendritic cells. Wild-type vesicular stomatitis virus (VSV) was a gift from Douglas Lyles (Wake Forest University School of Medicine) and was grown as previously described (Lyles et al., 1996).

DC infection and treatments

Virus infections and treatments were performed directly in the wells where the BMDC were generated to avoid maturation of cells as a result of manipulation. VV-Ova was diluted in RPMI and added to the BMDC cultures at a multiplicity of infection (MOI) of 0.1, 1, or 10 PFU/cell. BMDC treated with 300 ng/ml lipopolysaccharide (LPS) (Sigma, St. Louis, MO) were used as a positive control for fully matured cells. UV inactivation of virus was carried out using the standard 254 nm, 115-V, 1A lamp (Ultra Lum, CA) for 20 min at a distance of 6.5 cm. These conditions destroyed all detectable infectious

units. For infection of CD8⁺ DC, cells were cultured overnight (12 h) in the presence of 100 ng/ml rGM-CSF to increase viability (Rizzitelli et al., 2005).

Cell staining and flow cytometry

At various times following treatment, VV-Ova-infected, mock-infected, and LPS (300 ng/ml)-treated DC were stained with the following antibodies: allophycocyanine-conjugated anti-CD11c together with phycoerythrin-conjugated anti-CD40, anti-CD80, or anti-CD86 antibodies (PharMingen, San Diego, CA). The cells were blocked with Fc block prior to staining to prevent nonspecific binding of antibodies. Flow cytometry was performed using a FACSCalibur and the data analyzed with CellQuest Pro software (both from BD, San Diego, CA).

Cytokine detection

Supernatants from BMDC cultures that were VV-Ova-infected, mock-infected, or LPS-treated were analyzed at various times following treatment for the presence of cytokines. Interleukin-(IL)-12p40 was detected by capture enzyme-linked immunosorbent assay (ELISA). IL-12p70, tumor necrosis factor alpha (TNF- α), IL-10, IL-6, IFN- γ , and MCP-1 were detected using the cytometric bead array (CBA) kit as per the manufacturer's instructions (BD, San Diego, CA).

IFN- α/β was measured using the VSV-based IFN- α/β bioassay. This assay measures the amount of IFN- α/β present based on the decreased susceptibility of L929 cells to VSV-mediated cytolysis. Briefly, infectious virus in the supernatants from BMDC cultures was inactivated at 4 °C overnight by acid treatment at pH 2, the acid was neutralized, and serial dilutions were incubated with L929 cells in 96-well plates overnight at 37 °C. As a standard, cells were incubated with serial fivefold dilutions of IFN (Universal IFN- α/β ; PBL Biomedical Laboratories, New Brunswick, NJ). The samples were aspirated, and cells were challenged with wild-type VSV at an MOI of 8 in 100 μ l of medium. Controls included cells infected with wild-type VSV alone and cells that were not challenged with wild-type VSV. Cells were incubated overnight at 37 °C, medium was aspirated, and cells were fixed with 95% ethanol. Cells were then stained with a 0.1% crystal violet solution in methanol. Absorbance was read at 540 nm on an ELISA reader (Ahmed et al., 2003).

Assessment of naïve T-cell proliferation and function

On day 6 post-culture, BMDC were mock-treated, LPS-treated, UV-VV-Ova-treated, or infected with VV-Ova. At the indicated times following treatment, the cells were pulsed with (LCMV) gp_{33–41} peptide. These cells were then washed to remove soluble peptide. Spleens were harvested from P14 mice (Rag^{-/-}) and processed to yield single cell suspensions. Splenocyte populations were at least 88% CD8⁺ T cells. CFSE (5 μ M) (5- and 6-carboxyfluorescein diacetate, succinimidyl ester, Molecular Probes, Eugene, OR)-labeled, antigen-specific P14 LCMV gp_{33–41}-specific TCR transgenic splenocytes were

then co-cultured with DC pulsed with gp_{33–41} peptide for 3 days at a ratio of 10:1 at 37 °C in 96-well plates. Alternatively, CFSE-labeled P-14 gp_{33–41}-specific TCR transgenic splenocytes were co-cultured with DC matured with LPS and either infected with VV-gp_{33–41} or pulsed with gp_{33–41} peptide for 3 days at a ratio of 10:1. On day 3 of culture, cells were harvested and stimulated with 10^{−6} M peptide for 5 h in the presence of GolgiPlug (BD PharMingen, San Diego, CA). Cells were then stained with anti-CD8 antibody, fixed and permeabilized, and IFN production assessed by intracellular staining. Proliferation was determined by the decrease in CFSE intensity as measured by flow cytometry. Analysis and quantitation of data were performed using FlowJo software (FlowJo, Ashland, OR).

Assessment of antigen presentation

On day 6 post-culture, BMDC were mock-treated, LPS-treated, or infected with VV-Ova. Twenty-two hours following treatment, the cells were pulsed with (LCMV) gp_{33–41} peptide. These cells were then washed to remove soluble peptide and co-cultured at a CTL to DC ratio of 2:1 for 5 h with a gp_{33–41}-specific CTL line in the presence of GolgiPlug (BD PharMingen, San Diego, CA). Cells were then stained with anti-CD8 antibody, fixed and permeabilized, and IFN production assessed by intracellular staining.

Statistical analysis

A paired Student's *t* test was used to compare significance of individual time points and BMDC treatments. *P* ≤ 0.05 was considered statistically significant.

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